Confrontando argumentos a favor y en contra de antígenos propios y ajenos en la evolución de la subpoblación estereotipada #4 en LLC

Dueling selections for and against auto and foreign antigens in the evolution of CLL stereotyped subset #4

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Introduction

The unique features of the BCRs expressed by CLL B cells have led to the hypothesis that BCR-mediated signaling promotes the transformation of a normal B lymphocyte to a CLL B cell[1-3]. This is best illustrated by the ~33% of CLL patients grouped into stereotyped subsets based on HCDR3 similarity and the use of similar IGHV-D-J rearrangements[4-6], leading to the assumption that B cells from each subset encountered distinct sets of shared antigenic epitopes that led the way to leukemia. In light of this, a number of autoantigens and exoantigens have been found to bind to CLL BCRs[7-10] and hence could be involved in the survival and proliferation of CLL B-cell clones. However, this view needs to be reconciled with the finding that CLL BCRs appear to universally undergo homotypic interactions ("self-association") and consequently lead to BCR-mediated signaling in the absence of additional non-IG (self)antigens ("autonomous signaling")[11,12]. The relationship of these two types of specificities (non-self-IG and self-IG) will be discussed and their effects on the development of the structural elements of the BCRs from a specific stereotyped subset delineated by focusing on stereotyped subset #4.

CLL clones in subset #4 bear *IGHV4-34/IGHD5-18/IGHJ6* and *IGKV2-30/IGKJ2* rearrangements and are always class-switched to IgG and somatically mutated[13,14]; they also often exhibit amino acid re-

placement mutations at defined positions within the antigen-binding site[15]. Hence, this subset provides an opportunity to study the influences of a specific *IGHV* genes and recurrent, restricted somatic mutations on selection for receptor structure and antigen specificity.

Binding capacities of subset #4 mAbs to (auto) antigens

We evaluated the (auto)antigenic specificities of subset #4 using 3 recombinant mAbs belonging to this stereotyped group, demonstrating that these mAbs bind to two antigenic epitopes: [1] those on the surface of viable memory human B cells and on B-cell line cells as well as on naïve B cells[16], and [2] those on the hemagglutinins of influenza A viruses. Moreover, we have documented that the same subset #4 mAbs do not bind to two usual targets of IGs using the *IGHV4-34* gene: i/I-type blood group glycans[17] and ss- and ds-DNA[18].

Although we have not yet definitively identified the nature of the surface antigen on viable memory B cells and on B-cell line (Ramos) cells, our findings indicate that this is distinct from that on the surface of naïve B cells bound by non-subset #4 Abs that use the *IGHV4-34* gene[19].

Regarding the reactivity with influenza hemagglutinins, we have found that this is directed to two sets of hemagglutinins belonging to phylogenetically distinct sets, indicating that subset #4 IGs bind to an epitope shared by multiple influenza viruses and hence can be considered "broadly-reactive". Moreover, subset #4 IGs were able to protect target cells from influenza virus infection. Notably, influenza viruses expressing these two types of hemagglutinins have been responsible for several pandemics over the past 70 years. Thus, since CLL is a disease of aging, it is not unreasonable to postulate that repetitive, temporally-spaced encounters with influenza viruses expressing these hemagglutinins were involved in the evolution of the selection of those normal B cells that eventually transformed to CLL.

Finally, since our preliminary findings suggest that the viable memory B-cell surface epitope is a carbohydrate and since the immunogenetic epitopes on viral hemagglutinins are usually carbohydrate in nature, it is also not unreasonable to postulate that the same glycan epitope is shared by these two targets. This is provocative in light of the fact that other non-IGHV4-34^{+} Abs can react with blood group glycans. Also intriguing is the finding that subset #4 IGs lack reactivity with I-/i- carbohydrate antigens, despite having an intact binding site in FR1 of *IGHV4-34* gene classically for such binding.

The impact of CSR on the structure, antigen-binding properties, and function of BCRs

It is well accepted that IG heavy (H) chain class switching provides a mechanism to change effector function. However, a few studies have suggested that CSR can also affect the degree of antigen reactivity and even specificity[20,21]. This is of particular interest since subset #4 IGs are always class switched to IgG. To understand the impact of class-switching on the specificity of subset #4 BCRs, all 3 CLL subset #4 variable domains were recombinantly expressed with a Cμ region, and their capacity for self-association and binding to the aforementioned antigens were evaluated.

In contrast to IgG molecules, the surface membrane IgM molecules were not capable of inducing cell autonomous signaling; this was independent of the presence/absence of somatic mutations. This suggests that the original subset #4 B cells underwent CSR and thereby developed autonomous signaling. Moreover, in contrast to subset #4 IgGs, Cμ-linked subset #4 molecules lost reactivity to viable B cells and to influenza A virus; due to the latter, these lost the capacity to protect from influenza infection. These findings indicate that the IgG isotype is required for binding to these antigens. Strikingly, a non-self-associable mutant of subset #4 IgG did not bind to viable B cells or influenza A virus, suggesting that the recognition of these antigens by subset #4 IgGs requires an IG - IG complex formed by self-association.

In this setting, the stability of the self-associated "receptor/antigen" complex is important. Therefore we addressed this by testing the stability of the surface expressed homodimer by probing with soluble subset #4 IgGs. When the surface expressed IgGs were exposed to the soluble IgGs, no interaction occurred. Thus, the homodimer forms stable, relatively long-lived complexes that could provide a stable binding site for the antigens on viable B cell surfaces and influenza viruses. Once the nature of the antigens on these two targets is identified, co-crystallization studies will be performed to address this possibility.

In contrast to wild type subset #4 mAbs that exhibited minimal binding to DNAs and showed no binding to I-/i- carbohydrate sequences, switching the Cγ region back to Cμ region dramatically increased the binding to both types of antigens.

Collectively these results demonstrate that IG isotype determines antigen specificity and reactivity for subset #4 IGs. CSR impacted specificity in 3 ways. First, it created a binding site for IG self-association that led to the ability to signal in the absence of foreign antigen. Second, it permitted the creation of a scaffold for a new type binding site not involving the original variable domains. Third, it helped reduce and escape from autoreactivity. Thus, for subset #4 IGs, isotype switching provided the ability of an autoreactive naïve B cell clone to survive and proliferate, by developing structural elements that are beneficial and tolerated.

Impact of somatic mutations on the structure and antigen-binding properties of subset #4 BCRs

The formation of a self-associating subset #4 IG does not involve those amino acids typically generated by SHM[12]. Hence, these recurrent somatic mutations suggest selection by antigen(s) other than self-IG.

By reverting somatic mutations back to their germline amino acids, we found that the development of binding to viable memory B-cell surfaces is independent of somatic mutations in the H chain variable domain. This was always the case regardless of which individual mutation was reverted, including the stereotyped "E28G". Instead, binding was created by a single, CLL subset #4-biased somatic mutation "N66D" immediately adjacent to VL CDR2 position of *IGKV2-30*[16]. Reverting "D66" back to its germline amino acid "N" disrupted binding to memory B cells, without impacting binding to naïve B cells. This supports our hypothesis that the antigens/epitopes on naïve and memory B cells recognized by subset #4 IGs are distinct.

However, somatic mutations had a different impact on binding to influenza virus. The L chain somatic mutation made only a moderate contribution to this binding, whereas the introduction of somatic mutations to the sequences outside of *IGHV4-34* gene more strikingly increased reactivity to flu virus. However, although wild-type subset #4 mAbs and the germline configuration (IgG) did not bind DNA, introduction of the somatic mutations outside of *IGHV4-34* dramatically increased such binding. This, however, was reduced by the introduction of the stereotype mutation "G28E" in the *IGHV4-34* gene, and completely eliminated when other non-stereotyped mutations were introduced. Eliminating this strong autoantigen binding, however, significantly compromised (20-fold less affinity) the collective increased anti-HA activity that somatic mutations made in *IGHV4-34* genes.

Together, these data suggest that BCR engagement with influenza virus led to somatic mutations and their selection. However this selection also led to unacceptable autoreactivity, which when eliminated by additional mutations, compromised reactivity to influenza.

Hypothetical model for the evolution of subset #4 BCRs

In summary, these findings indicate that the original subset #4 BCR contained germline-like *IGHV4-34/IGHD5-18/IGHJ6* and *IGKV2-30/IGKJ2* rearrangements linked to a Cμ region. These BCRs were autoreactive, binding to DNAs and i-type glycans. However the autoreactivities were significantly reduced (in case of i-type antigens) or completely eliminated (in case of DNAs) by CSR to IgG. Class-switching to IgG also resulted in IgG self-association, which consequently induced autonomous signaling.

Meanwhile/concomitantly, the "N66D" somatic mutation in the L chain rearrangement enabled binding to viable B-cell surfaces and mutations made outside *IGHV4-34* on the H chain rearrangement increased reactivity to influenza. However, the same mutations re-enabled strong binding to DNAs, albeit due to a different set of residues than in the original rearrangement, and this was reduced by the stereotyped mutation "G28E" on *IGHV4-34* and was diminished further by other mutations made throughout this gene. These collective mutations impaired considerably the affinity for influenza virus but made any residual autoreactivity tolerable.

Although we cannot be sure of the relative timing of the effects that CSR and SHM had on BCR structure and function, overall these findings illustrate the importance of positive and negative selection for the structural changes in CLL subset #4 IGs that ultimately determined the unique antigen-reactivities of these CLL B-cell clones.

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